

Rosiglitazone Role in the Expression of KLF6 Caco-2 Colon Cancer Cells

Rol de Rosiglitazona en la Expresión de KLF6 en Células Caco-2 de Cáncer de Colon

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SUMMARY: Kruppel-like factor 6 (KLF6) is a member of the family of Kruppel transcription factors, this plays an important role in the regulation of cell growth, differentiation and angiogenesis. Rosiglitazone is a PPAR γ agonist drug, its antitumor effect has been described in models of breast and colon cancer. The aim of this study is to evaluate the level of expression of KLF6 in Caco2 cells treated with Avandia. For this a Immunofluorescence was performed, the Caco2 cells were cultured and treated with Rosiglitazone, another group was treated with Rosiglitazone and GW-9662, inhibitor for Immunofluorescence an anti-KLF6 antibody and a secondary antibody coupled to Alexa-488 was used. Cells were observed in a fluorescence microscope and images were processed. The results show that KLF6 is expressed in the cytoplasm of cells Caco2. Compared to treatment with Avandia, KLF6 increases its expression in the cytoplasm. When cells were treated with GW-9662 inhibitor, an expression of KLF6 in the nucleus was observed. KLF6 expression in the cytoplasm of cells Caco2, could be explained by the knowledge of splicing variants SV1 and SV2, these abnormally accumulate in the cytoplasm and promotes cell growth. It is concluded that in untreated Caco 2 cells, KLF6 is expressed in the cytoplasm. Compared to treatment with Rosiglitazone, KLF6 upregulated in the cytoplasm and compared to treatment with the inhibitor, KLF6 is expressed in the nucleus of Caco 2 cells.

KEY WORDS: KLF6; Rosiglitazone; Colon and KLF6.

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide and the third leading cause of death from cancer (Ferlay *et al.*, 2015). Risk factors associated with colorectal cancer include consumption of alcohol, snuff, diets rich in fat, high consumption of red meat and sedentary lifestyle. Factors that could lead to states of obesity (Johnson *et al.* 2013). In addition, other factors such as hereditary, has shown that personal or family history of colorectal cancer have a risk in the development of the disease, and people with development of adenomatous polyps in the colon (Corley *et al.*, 2014). There is also a high association between chronic inflammatory bowel diseases, such as ulcerative colitis (UC) and Crohn's disease (CD) in the development of colorectal cancer (Kisiel *et al.*, 2013; Ryan *et al.*, 2014).

KLF6 is a protein belonging to the family of Krüppel factors or transcription KLFs type, this transcription factor is characterized by three DNA binding domains, called zinc fingers. KLF6 has great importance in the control of various processes as cell growth, cell differentiation, apoptosis and

angiogenesis (Gehrau *et al.*, 2011; Lang *et al.*, 2013). In relation to its function, it is known that KLF6 is a tumor suppressor, also inactivation either by loss of heterozygosity (LOH), somatic mutation or methylation of its promoter, leads to the development of various types of cancers such as prostate, colon, gliomas and hepatocellular carcinoma (Di Feo *et al.*, 2009).

Another mechanism that promotes the development of various types of cancers, and probably colon, is through the expression of alternative splicing variants of KLF6, as SV1 and SV2, these splice variants are located abnormally in the cytoplasm, because they have lost their nuclear localization sites (Vetter *et al.*, 2012; Narla *et al.*, 2005). Furthermore, studies on the expression of SV1 in Prostate Cancer lines found lowered expressions of p21 and increased cell growth (Narla *et al.*, 2005). Because KLF6-SV1, lacks the binding domain DNA, called "zinc fingers", there is no transactivation of p21 and E-cadherin genes, which are required for the KLF6 tumor suppressor function (Narla *et al.*, 2001; DiFeo *et al.*, 2006).

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PPAR γ (peroxisome proliferator-activated receptor), belongs to a family of nuclear receptors, mediates the effects, level control of gene expression, steroid hormones, glucocorticoids, thyroxine, retinoic acid receptor and vitamin D. There are three variants derived from PPAR transcriptional gene called γ 1, γ 2 and γ 3. It was initially identified as an important factor in the formation and differentiation of adipocytes (Voutsadakis *et al.*, 2007). In relation to colon cancer, it has been found that PPAR γ is expressed in the normal colonic epithelium, whereas the decrease in the expression of this nuclear receptor, can increase the risk of developing colon cancer (Youssef & Badr, 2011; Pancione *et al.*, 2010; Qi *et al.*, 2009).

Regarding the PPAR agonists, the most important is Rosiglitazone is a drug belonging to the family of thiazolidinediones. Rosiglitazone is currently used for the treatment of type II diabetes (Chiu *et al.*, 2010) there has also been described the antitumor effect of this drug, inhibits the endothelial growth and angiogenesis in models of breast cancer, and induces apoptosis in the cell cycle in colorectal cancer cells (Aires *et al.*, 2014; Bieker, 2001).

MATERIAL AND METHOD

Cell culture. Cell lines of human colon adenocarcinoma Caco-2 (Sigma-Aldrich) were used, they were maintained in Dulbecco Modified Eagles Medium, DMEM (Sigma-Aldrich) supplemented with 20 % fetal bovine serum (FBS) and 1 % penicillin / Streptomycin. The Caco-2 cell lines were maintained at 37 °C in incubator with 95 % humidity and 5 % CO₂. For planting and cultivation 1X Trypsin-EDTA (Sigma-Aldrich) was used. For treatments, cells were seeded in 24-well plates for culture, until a cell confluency of 70 %, then, the observations were made under an inverted microscope.

For viability and cell count Trypan Blue was used and an automated cell counter, Bio-Rad Tc 20 Automated cell counter. DPBS (Dulbecco's phosphate-buffered saline) (Sigma-Aldrich) was used for crop washings before treatments.

Treatment and reagents. PPAR γ agonist drug Rosiglitazone was used (Sigma-Aldrich) and rosiglitazone inhibitor, GW-9662 (Sigma-Aldrich). Furthermore rabbit polyclonal antibodies against KLF6 (Santa Cruz Biotechnology) were used, and the secondary antibodies goat anti-rabbit coupled to Alexa-488 (Santa Cruz Biotechnology) were used. For nuclear staining, it was used 4,6-diamino-2-phenylindole (DAPI) (Sigma-Aldrich), for washing 1x PBS (Sigma-Aldrich) was used.

Management cell culture. Cell lines Caco-2 were incubated for 24 hours, then the cells were seeded in an amount of 1X

10⁶ cells per ml, using 24-well plates for incubation, with daily observations in an inverted microscope, for verification and scope confluency.

Rosiglitazone treatment. After reaching the required confluence, Caco2 cell lines, DPBS were washed with 1x (Sigma-Aldrich). The cells were subsequently treated, the first group with 20 μ M rosiglitazone (Rosi) (Sigma-Aldrich) in the first 4 plates, the second group with a mixture of Rosiglitazone (20 μ M) and the inhibitor of Rosiglitazone, GW-9662 (10 μ M) (Sigma-Aldrich) in the next 4 plates and the third group correspond to untreated cells that were left as expression control KLF6. They were incubated with 500 μ L of complete DMEM medium (Sigma-Aldrich) at 37 °C for 24 hours.

Immunofluorescence assays. Caco2 cell lines were fixed with paraformaldehyde 4 % sucrose (Sigma-Aldrich) for 20 minutes at room temperature. Then washed with 1x PBS (Sigma-Aldrich) for 5 min, then cells were permeabilized with PBS-Triton X-100 0.1 % (Sigma-Aldrich) for 5 minutes, again washed with 1x PBS (Sigma-Aldrich), then treated with a blocking PBS-1 % BSA solution (Sigma-Aldrich) for 1 hour. Then the cells were incubated with anti-KLF6 (Santa Cruz Biotechnology) primary antibody, at a concentration of 1: 100 in PBS-BSA (Sigma-Aldrich), incubation was performed at 4 °C overnight. The cells were washed three times with 1x PBS (Sigma-Aldrich), and incubated with secondary antibody goat anti-rabbit coupled to Alexa-488 (Santa Cruz Biotechnology) at a concentration of 1: 250 in PBS-BSA (Sigma-Aldrich), and incubated at 37 °C for one hour for nuclear staining (DAPI) was used for 10 minutes at room temperature, then they were mounted with Fluoromont. Finally, the cells were observed in fluorescence microscopy Olympus IX 81.

Immunofluorescence images, captured for nucleus with DAPI and KLF6 were taken separately, then images were co-located using the Image J software v1.49 (<http://imagej.nih.gov/ij>), using the Merge function.

RESULTS

KLF 6 Expression in Caco 2 cells. The results show that in Caco2 cell lines not treated and observed to microscopy immunofluorescence show that KLF6 is apparently expressed a strong expression in the nucleus (Fig. 1A), however cell groups are also observed, with increased expression of KLF6 in the cytoplasm.

KLF6 expression in Caco 2 cells treated with PPAR γ . The results obtained in the Caco-2 cell cultures, following

treatment with rosiglitazone, a marked increase in the expression of KLF6, in the cytoplasm (Fig. 2A), greater than in the untreated was observed.

KLF6 expression in Caco 2 cells treated with

Rosiglitazone and its GW-9662 inhibitor. The results in Caco2 cell lines, observed by fluorescence microscopy showed that the expression of KLF6 is increased mainly in the nucleus of the cells (Fig. 3A) a slight decrease is observed in the cytoplasm.

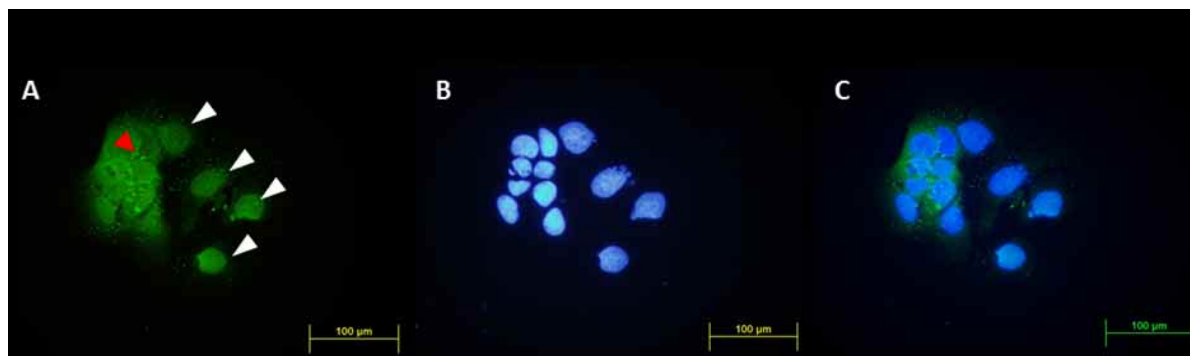


Fig. 1. Expression and cellular localization of KLF6 by Immunofluorescence in Caco2 cells. A) culture cells Caco-2 with nuclear expression of KLF6 (white arrows) and cytoplasmic, the latter with granular aspect (red arrow) are observed. B) The cell nuclei were labeled with DAPI (blue). C) image overlay A and B. The total magnification of the image was 400 x 100 microns Scale bar.

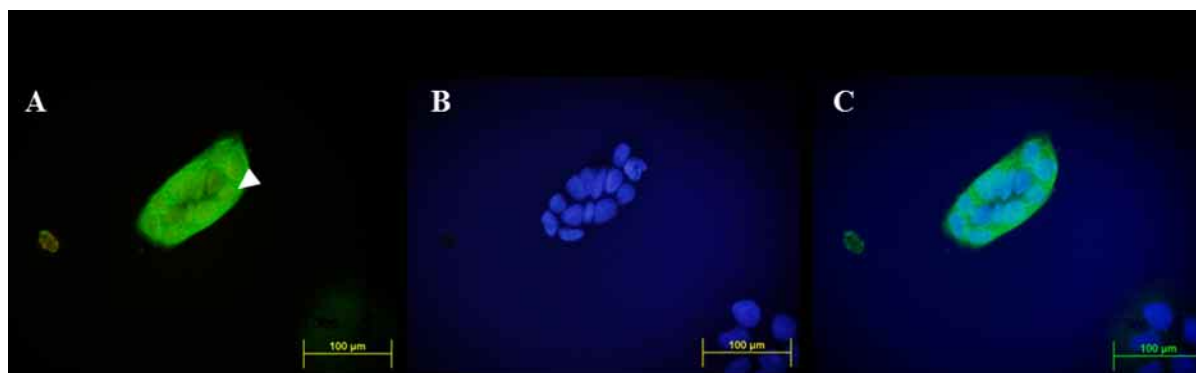


Fig. 2. Changes in the expression of KLF6 in Caco-2 cells treated with Rosiglitazone (Rosi). Cells were treated with 20 μ M Rosiglitazone and analyzed by immunofluorescence. A) an increase in expression is observed KLF6, localized predominantly in the cytoplasm of cells (white arrow) B) Cell nuclei were labeled with DAPI (blue). C) of the above superposed image. The total magnification of the image was 400 x 100 microns Scale bar.

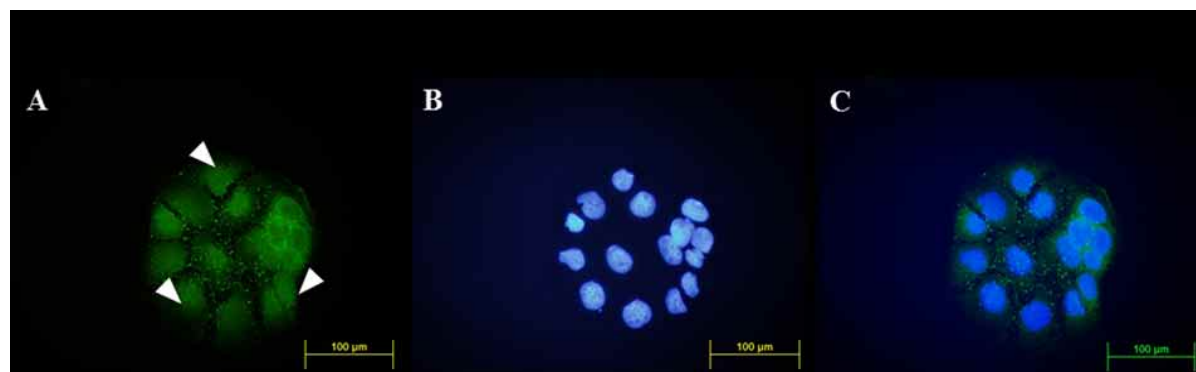


Fig. 3. Effect on KLF6 expression compared to treatment with Rosiglitazone inhibitor GW- 9662. The Caco-2 cells were co-treated with Rosiglitazone (Rosi) 20 μ M 10 μ M more inhibitor GW-9662. KLF6 expression was analyzed by immunofluorescence. A) KLF6 expression is observed mainly in the nucleus of cells (white arrows) (B) Cell nuclei were labeled with DAPI (Blue) C) superimposed image of the above. The total magnification of the image was 400 X. Scale bar 100 μ m.

DISCUSSION

KLF6 plays a key role in cell cycle regulation, differentiation and apoptosis (Lang *et al.*; Bieker; Mallipattu *et al.*, 2015). Furthermore, it has been shown that functional inactivation of KLF6 in various types of cancers such as prostate, ovary and colon, is caused by somatic mutations, loss of heterozygosity (LOH) and silencing by hypermethylation of its promoter (DiFeo *et al.*, 2009; Cho *et al.*, 2006; Sangodkar *et al.*, 2009).

Another deregulating mechanism tumor suppressor activity of KLF6 is the production of alternative splicing variants, mutated KLF6, produces alterations in processing sites of messenger RNA, resulting in aberrant protein isoforms KLF6, losing tumors suppressor function (DiFeo *et al.*, 2009). In studies in patients with prostate cancer, alternative splicing variants of KLF6, SV1 and SV2 were found, finding forms of alternative processing than wild forms of KLF6, this correlates with poor prognosis in patients with Prostate Cancer (Narla *et al.*, 2005; Narla *et al.*, 2008).

Regarding the subcellular location of KLF6, in Caco2 cells, it has been found that it is primarily expressed in the cytoplasm, suggesting a possible form of expression Alternative KLF6 splicing (Fig. 1A). In accordance with the aforementioned, it has been found that KLF6- SV1 and SV2 variants, are expressed and are located in the cytoplasm, and this location determines its promoter character of cell growth and metastatic processes in Prostate cancer (Narla *et al.*, 2005; Narla *et al.*, 2008). The expression of KLF6 cytoplasmic, has also been found in some Caco2 cells of the present study (Fig. 1A), these results are similar to studies on KLF6-SV1 in Prostate Cancer, these results describe the cytoplasmic distribution of KLF6, this it is due to the absence of the nuclear localization signal in the protein (Narla *et al.*, 2005).

In 2010, Rodríguez *et al.*, found mutations in M6 and R243 amino acid of the nuclear localization domain of KLF6, such mutations determined its translocation into the cytoplasm, also it was found that the sequence of nuclear signaling is located within one of the domains of the KLF6 zinc finger, called site ZF1 (zinc finger 1), in this sense KLF6-SV1 lacks the nuclear localization sequences, losing its functionality in the core and accumulate thus in the cytoplasm (Rodríguez *et al.*, 2010).

Rosiglitazone is an agonist of PPAR γ , ie it induces its expression, and also has been shown to have an antitumor effect by inhibiting processes as angiogenesis,

growth and induction of apoptosis in colorectal cancer cells (Chiu *et al.*; Gupta & Dubois, 2001). It has also been shown that KLFs transcription factors, can regulate the expression of PPAR γ producing cells increased lipogenesis and adipogenesis. Specifically it has been found that proteins klf9 KLF6 and PPAR γ regulate the increased expression of this, it promotes the development of hepatic steatosis, condition in which triglycerides accumulate in the hepatocytes (Youssef & Badr).

The effect of the PPAR γ agonist drugs, such as rosiglitazone, in Caco-2 cells, when treated, was observed an increased expression of KLF6 in the cytoplasm (Fig. 2A). These results might suggest that they would be expressing perhaps other forms of alternative processing of KLF6, losing the ability to act in the nucleus accumulate in the cytoplasm, this could lead to suggest a disruption yet undescribed on malignant characteristics of Caco2 cells, through the expression of other forms of alternative splicing of KLF6. However, other studies have described the antiproliferative effect of Rosiglitazone in Colon cancer cells, linking Rosiglitazone processes with the stop in cell growth and apoptosis (Pancione *et al.*; Aires *et al.*), not to mention the location of KLF6, and its effects on cell proliferation.

Furthermore, the effect on the expression of KLF6 in the cytoplasm by treatment with Rosiglitazone inhibitor GW- 9662. After treatment with the inhibitor, KLF6 was observed with a clear expression in the nuclear area of the cells (Fig. 3 a), this could prove Rosiglitazone involved in the expression level of KLF6 at a cytoplasmatic level in Caco2 cells. These preliminary results are not sufficient to demonstrate the inhibitor power of GW-9662.

CONCLUSION

The preliminary results show that Rosiglitazone, PPAR γ agonist, induces the expression of KLF6 localized mainly in the cytoplasm of Caco-2 cells, suggesting the existence of an unknown mechanism, in which they would be expressing forms of Splicing alternative KLF6 at a cytoplasmatic level, in the cell lines of colon cancer cells Caco2.

We show apparently that PPAR γ agonist drugs as rosiglitazone, might be able to inhibit the growth in cell lines as Caco2 colon cancer. In this preliminary

investigation it has been shown that increases its cytoplasmic KLF6 when treated with agonists PPAR γ expression in Caco2 cells, suggesting a direct relationship between PPAR γ and KLF6. The cytoplasmic localization of KLF6, could show the existence of other forms of alternative splicing of KLF6, suggesting the possible deregulation of other proteins related to cell proliferation.

On the other hand, treatment with rosiglitazone inhibitor GW-9662, shows a slight decrease in the expression of KLF6 cytoplasmic level, evidenced mainly in the nucleus of Caco2. They should continue to make further assessment with PPAR γ agonists on the Caco2 cells to elucidate the actual effect of the drug on KLF6 and variations of changes in their expression.

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KEY WORDS: KLF6; Rosiglitazone; Colon; KLF6.

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